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Rebound Swelling of Astroglial Cells Exposed to Hypertonic Mannitol

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Abstract

Background: Mannitol is widely used in anesthesia and critical care medicine. Although its clinical effects were originally attributed to osmotic dehydration of brain cells, other mechanisms have also been proposed. Osmotic dehydration of astroglial cells is opposed by powerful volume-regulating mechanisms that involve inward transport of electrolytes. These mechanisms have been studied previously by exposing cells to hypertonic saline gradients. Because of its potential clinical relevance, the volume response of astroglial cells exposed to hypertonic mannitol was investigated.

Methods: Rat C6 glioma cells were cultured to confluence, and their volume behavior was observed by laser light scattering. After equilibration at physiologic temperature and pH, cells were abruptly exposed to hypertonic mannitol solutions. In separate experiments, C6 cells were exposed to

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hypertonic solutions containing radiolabeled mannitol, and its cellular uptake was determined.

Results: Hypertonic mannitol exposure produced initial cell shrinkage followed by rapid volume recovery and rebound swelling. The rebound swelling was similar in magnitude to the initial maximal shrinkage. For +40 mOsm and +70 mOsm mannitol challenges, mean volume recovery was 184 +/- 31% and 227 +/- 62%, respectively (where full recovery to baseline volume = 100%). Rebound swelling was substantially inhibited by furosemide. When exposed to mannitol in varying concentrations, uptake was linear, ranging from 82 +/- 7 nmol/mg to 406 +/- 26 nmol/mg protein. After 5 min, estimated intracellular concentrations of mannitol were similar to extracellular concentrations.

Conclusions: Unlike hypertonic saline, hypertonic mannitol exposure produces rebound cell swelling. Cellular penetration of mannitol appears to account for much of this phenomenon. The clinical implications of these observations remain to be determined.

This article is featured in "This Month in Anesthesiology." Please see this issue of Anesthesiology, page 5A.

Key words: Brain, cell volume; mannitol; osmotherapy; rebound.

SINCE its introduction more than 30 yr ago, osmotherapy with mannitol has been a mainstay of neuroanesthesia and critical care medicine. [1] Osmotic, [2] rheologic, [3-5] hemodynamic, [5-7] and cellular [8] mechanisms have been advanced to account for the clinical effects of mannitol therapy. Previous investigations into potential osmotic mechanisms have shown that astroglial cells vigorously resist osmotic shrinkage by rapidly activating electrolyte transport processes collectively aimed at restoring cell volume. [9] Such acute volume regulation, called regulatory volume increase (RVI), is a basic homeostatic mechanism common to many cell types. [10,11]

Although it is generally thought that mannitol remains in the intravascular space, it can enter the central nervous system under a variety of circumstances when the integrity of the blood-brain barrier is compromised. In the setting of severe neurologic injury, in which therapy for cerebral edema is most important, mannitol may enter the brain and directly bathe cells of the central nervous system. Although astroglial cells have been shown to maintain constant volume during hypertonic saline exposure, the response to hypertonic mannitol has not been described. The following series of experiments was conducted to characterize this response.

Materials and Methods

Cell Culture

Rat C6 glioma cells, purchased from the American Type Culture Collection (Rockville, MD), were cultured in Eagle's minimal essential medium with 10% fetal bovine calf serum and penicillin - streptomycin. Cultures were maintained in a humidified 5% carbon dioxide and 95% air atmosphere at 37 [degree sign]C, and growth media was changed every 48 h. Cells

were seeded at low concentrations and all experiments were conducted after 3 - 5 days, when cultures had reached approximately 80 - 90% confluence. Before each experiment, microscopic examination confirmed the presence of healthy cells with normal structures.

Experimental Solutions

For laser experiments, isotonic (290 mOsm) control solution was buffered in bicarbonate (pH 7.4) and composed of 5.4 mM KCl, 116.4 mM NaCl, 26.2 mM $NaHCO_3$, 1 mM $NaHPO_4$, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 5.5 mM glucose, 2 mM glutamine, and 0.02 mM inositol with 30 nM selenium, 10 mg/l biotin, 5 mg/l insulin, 10 nM hydrocortisone, 5 pM tri-iodothyronine, and 25 mg/l prostaglandin E_1. Hypertonic (330 and 360 mOsm) solutions were produced by adding mannitol, and osmolarities were measured by freezing-point depression (Osmette A, Precision Systems, Sudbury, MA). For inhibitor experiments, drug was added to both control and experimental solutions.

Flux solutions used in [(^{3}) H]mannitol uptake experiments were buffered with HEPES, pH 7.4, and contained 10 mM HEPES, 143 mM NaCl, 5 mM KCL, 1.2 mM $MgCl_2$, 0.02 mM inositol, and 2 mM $CaCl_2$. Solution osmolarities were confirmed as 290 +/- 5 mOsm, and then mannitol was added to desired concentrations. Stop solutions contained 150 mM NaCl, 10 mM HEPES, 1 mM quinidine, and mannitol to match experimental conditions.

Cell Volume Observation

Real-time observations of relative cell volume were made using laser light scattering, as described previously. [9,12] Briefly, cells were cultured as noted already to near-confluency on 11 x 22 mm rectangular glass cover slips, which were then mounted in a custom-designed observation cuvette. The cuvette was perfused continuously with experimental solutions maintained at 36 - 38 [degree sign]C in equilibrium with 5% carbon dioxide and 95% oxygen. A spot on the cover slip was then illuminated with a 5-mW red helium - neon laser (model 05-LHP-151; Melles Griot, Irvine, CA), and scattered light was detected by a photomultiplier tube (model 9826B; Thorn EMI, Fairfield, NJ). Photomultiplier tube voltage output was conducted sequentially through (1) a signal preamplifier and voltage converter (model A1; Thorn EMI), (2) a low-pass filter (model 902; Frequency Devices, Haverhill, MA), and (3) an A/D conversion board (model DT2821; Data Translation, Marlborough, MA) interfaced to a personal computer and sampled at 1 Hz. In this system, decreases in cell volume are detected as increases in light scattering and photomultiplier tube voltage output, and increases in cell volume are detected as decreases in light scattering and photomultiplier tube voltage output. In previous studies, volume fluctuations measured by laser light scattering were confirmed and quantified using light microscopic examination and digital image analysis techniques. [13] Although they yield no information regarding total cell volume, changes in light scattering linearly reflect changes in volume. Thus the system permits real-time quantification of the rate and extent of cell volume fluctuations. [12]

Experimental Protocol

In all experiments, cells were first equilibrated for 30 min under baseline conditions and the photomultiplier tube output was set to zero. Isotonic solution perfusate was then abruptly replaced with hypertonic experimental solutions and volume behavior observed as changes in scattered light intensity (voltage deflections from zero). Because loop diuretics have been

shown to substantially inhibit acute RVI in C6 cells exposed to hypertonicity, [9] the effect of furosemide on the response to hypertonic mannitol exposure was also investigated. For these experiments, cells were equilibrated in isotonic buffer containing furosemide (10^{-5} M) and then exposed to a solution containing both furosemide and mannitol (+40 mOsm). Four to six experiments were completed for each group.

[(^{3}) H]Mannitol Uptake Measurements

Because mannitol penetration and reversal of osmotic gradients may contribute to rebound cell swelling, the degree of mannitol influx during hypertonic exposure was investigated. Cells were cultured as before and grown to near-confluency in six-well dishes (GIBCO, Grand Island, NY), and flux measurements were performed as described previously. [13] Briefly, culture dishes were removed from the incubator immediately before experiments and each well was washed twice in 3 ml flux media. Cells were then incubated in an additional 2 ml flux medium at 37 [degree sign]C for 15 min. The medium was discarded and replaced with 2 ml medium that was made hypertonic by the addition of mannitol and 5 mCi/ml [(^{3}) H]mannitol (New England Nuclear, Billerica, MA). After a 5-min incubation period at 37 [degree sign]C, cells were rapidly washed three times with 3 ml ice-cold hypertonic stop solution and extracted overnight in 2 ml 0.25 N NaOH. Aliquots of cell extract were then taken for liquid scintillation and protein determination by Lowry assay.

Data Analysis

For analysis and graphic display, voltage data are presented as relative cell volume changes with steady state isotonic equilibration voltages defined as zero. Subsequent volume behavior is then described by reference to baseline with recovery considered 100% when baseline is restored, <100% when hypertonic shrinkage persists, and >100% when volume overshoot (swelling) occurs. Data summarizing several experiments are expressed as means +/- SD. Initial rates of recovery are expressed as the slope of regression lines drawn through the first 100 data points obtained during recovery ($R^2 > 0.95$). The effect of furosemide on volume response was analyzed using the Student's two-way t test to compare independent means.

Results

Exposure to Hypertonic Mannitol Causes Rebound Cell Swelling

Stable light scattering signals obtained in isotonic control solution were disrupted only momentarily (<10 S) by solution changes. Using blank cover slips as controls, the presence of mannitol in the concentrations used for these experiments did not itself produce a significant change in scattered light intensity.

As depicted in Figure 1, abrupt exposure to hypertonic mannitol (+70 mOsm, n = 4) initially produced rapid cell shrinkage. After a brief lag period, cell volume began to return toward baseline at a mean initial rate of 1.7 +/- 0.36%/s^{-1}. Cell volume then proceeded to increase beyond baseline, subsequently stabilizing in a swollen state for the duration of the 30-min observation period. Exposure to a smaller mannitol gradient (+40 mOsm, n = 5) resulted in similar behavior with nearly identical recovery rates (1.8 +/- 0.33%/s^{-1}) yet slightly less rebound swelling (Figure 2). Mean subsequent volume recoveries after +70 and +40 mOsm exposures were +227 +/- 62% and +184 +/- 31%, respectively. Assuming an osmotically active cell fraction of 80%, the shrinkage expected from a +70 mOsm hypertonic challenge can be estimated as 15 - 20%. In these experiments, this shrinkage would temporally coincide with the maximal voltage deflections seen shortly after mannitol

exposure. As they had a similar absolute value, the rebound voltages that we observed suggest subsequent cell swelling of 15 - 20%.

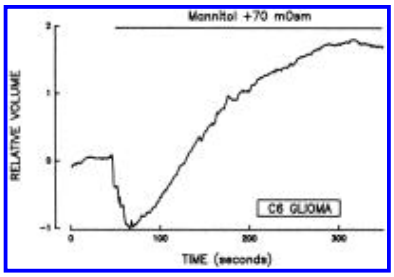

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Figure 1. A representative recording of the volume behavior of C6 cells exposed to +70 mOsm hypertonic mannitol. Cells were equilibrated in isotonic bicarbonate-buffered solution and then exposed abruptly to hypertonic conditions. Relative cell volume was observed by laser light scattering as described in the text.

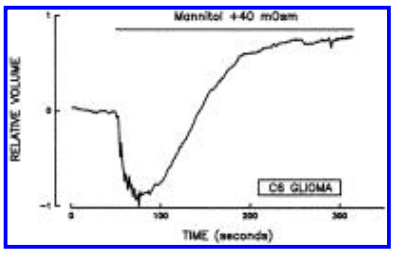

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Figure 2. A representative recording of the volume behavior of C6 cells exposed to +40 mOsm hypertonic mannitol.

As shown in Figure 3, rebound swelling was significantly inhibited by the presence of furosemide ($P < 0.001$). Cells exposed to mannitol in the presence of furosemide were observed to undergo rapid RVI, yet final equilibrium volumes returned close to baseline (recovery volumes = 90 +/- 7%, n = 5).

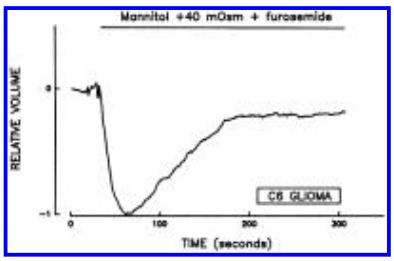

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Figure 3. Cells exposed to +40 mOsm mannitol in the presence of 10^{-4} M furosemide.

Mannitol Enters Cells under Hypertonic Conditions in a Concentration-dependent Manner

After a 5-min exposure of C6 cells to hypertonic solutions containing mannitol in concentrations of 25, 50, and 100 mM, [(^{3})H]mannitol was observed to enter cells (n = 4 at each concentration). Uptake of the radiolabel was linear ($R^2 = 0.9916$) and showed no sign of saturation at these concentrations (Figure 4). Total mannitol accumulation was 82 +/- 7 nmol/mg protein at

25 mM, 162 +/- 16 nmol/mg protein at 50 mM, and 406 +/- 26 nmol/mg protein at 100 mM. Assuming a final cell water content of 3 - 4 [micro sign]l/mg protein, [13-15] intracellular mannitol concentrations closely resembled extracellular concentrations.

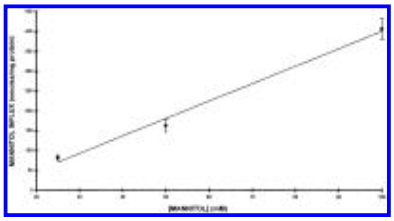

Figure 4. Mannitol content in C6 cells (y axis) after 5 min of exposure to solutions made hypertonic by adding mannitol in varying concentrations (x axis). Points are means +/- SD; n = 4.

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Discussion

We report the counter-intuitive observation that brain cells exposed to a hypertonic mannitol gradient ultimately swell far above their initial resting volume. This response is both qualitatively and quantitatively different from that of cells exposed to simple hypertonic saline, where little or no rebound swelling is observed. [9] Although somewhat unexpected, these observations are consistent with experiments in other models. For example, Kuncz et al. [16] observed that, for equal increases in plasma osmolality, reduction of brain volume with mannitol is less than that observed with hypertonic saline. In other investigations, Chebabo et al., [17] while trying to measure cell and interstitial volumes in rat hippocampal slices exposed to mannitol, obtained "erratic results" and speculated that glial cells may actually swell under such conditions.

The observations that a greater mannitol concentration produces greater rebound swelling and that the rate of RVI in mannitol solution is increased over previously reported recovery rates in hypertonic saline (1.8 vs. 1.1%/s^{-1}) [9] suggest concentration-driven entry of mannitol into cells. The [(^{3}) H]mannitol uptake measurements presented here support this. The amount of mannitol entering cells under these conditions corresponds closely to external mannitol concentrations. It is therefore not surprising that the extent of rebound cell swelling mirrors initial cell shrinkage. The degree of mannitol accumulation observed here is osmotically significant and would reasonably be expected to carry volume consequences. By comparison, C6 cells accumulate a similar organic solute, inositol, to levels of 450 - 500 nmol/mg protein during acclimatization from 290 - 440 mOsm. [13] This has been estimated to account for 50 - 75% of the solute required for complete volume regulation despite predicted osmotic shrinkage of >40%.

Kimelberg and Goderie [18] reported that mannitol may gain entry to the intracellular space during swelling of rat astrocytes, and they speculated that this occurs through large channels opened during cell swelling. In our investigation, mannitol entered the cells even under hypertonic conditions at which cell shrinkage was observed. Influx of mannitol may occur immediately on exposure or as the cell swells during RVI. The increases in initial RVI rates (obtained during the first 100 s of exposure) that we observed suggest that solute entry is early.

The finding that furosemide attenuates this response suggests that normal RVI processes generally, and the Na/K/2Cl cotransporter in particular, also participate in this phenomenon. Several potential mechanisms for this observation may

be considered. Solute entering via normal RVI processes may simply be additive with mannitol entry in production of rebound swelling. Alternatively, mannitol entry may be slow in shrunken cells and facilitated by volume recovery. Finally, in addition to its effect on other membrane transport processes, furosemide may directly block mannitol entry. This issue is the subject of ongoing investigations.

Because acute volume-regulatory processes appear to remain active, it is interesting that cells exposed to mannitol cannot volume regulate after rebound swelling. Typically, acutely swollen cells try to restore normal volume by losing solute and water via processes collectively called regulatory volume decrease. Because efflux of organic solutes is slow, this process is impaired in cells that have accumulated large concentrations of organic solutes, such as during chronic acclimation to hypertonicity. [13] We speculate that regulatory volume decrease was not observed in our experiments because accumulated mannitol cannot easily exit the cell against a concentration gradient.

The clinical relevance of these observations remains to be seen. Many investigators have reported rebound increases in intracranial pressure after mannitol administration and, in at least two animal studies, the curves of intracranial pressure versus time after mannitol treatment are, in fact, qualitatively similar to the curves of cell volume versus time that we described here. [5,19] However, the conditions that we used in our experiments are extreme and make even the most cautious clinical extrapolation difficult. Certainly even very large doses of mannitol in patients with massive disruption of the blood - brain barrier would be unlikely to produce direct exposure of brain cells to mannitol in the concentrations used here. It is unlikely, therefore, that the phenomenon reported here underlies the rebound intracranial hypertension sometimes observed clinically. We speculate that under certain conditions, such as with continuous infusion or repeated doses given over extended periods, the degree of mannitol transfer across the blood - brain barrier may become significant. If so, experiments here suggest that such entry of mannitol into the brain is potentially harmful.

Early investigations using radioisotopes demonstrated brain and cerebrospinal fluid penetration of mannitol in healthy animals such that 6-h brain tissue levels exceeded cerebrospinal fluid levels, although they still remained less than plasma concentrations. [20] Although human data are limited, highly variable penetration of mannitol into the cerebrospinal fluid has been described in neurosurgical patients, with cerebrospinal fluid concentrations varying by an order of magnitude 4 - 6 h after a single 1 g/kg bolus. [21,22] In a feline model of cryogenic brain injury, Kaufmann and Cardoso [23] observed that five doses of mannitol repeated at 4-h intervals led to significant accumulation in cerebral tissues with exacerbation of cerebral edema. However, other investigators, using a different model have been unable to confirm such deleterious effects. [24]

The data presented here suggest that direct exposure to significant concentrations of mannitol can interfere with normal cell volume regulation and lead to paradoxical cell swelling. Thus, in addition to extracellular accumulation with reversal of osmotic gradients, cell swelling itself may be considered a potential mechanism for exacerbation of cerebral edema during repeated or prolonged mannitol administration. This mechanism takes on added significance given that astrocytic swelling may promote secondary neurologic injury by stimulating release of excitatory neurotoxins. [25-27] Unfortunately, in clinical settings it is difficult to distinguish between evolving disease and iatrogenic exacerbation of brain swelling. Thus further understanding of the mechanisms involved here is required before clinical applicability can be determined. Expanded study of cell volume regulation in the brain may provide the tools necessary to optimize the clinical efficacy of osmotherapy.

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